Evaluation of matrix-assisted laser desorption ionization-time of flight mass spectrometry for species identification of *Acinetobacter* strains isolated from blood cultures

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Abstract

The clinical relevance of *Acinetobacter* species, other than *A. baumannii*, as human pathogens has not been sufficiently assessed owing to the insufficiency of simple phenotypic clinical diagnostic laboratory tests. Infections caused by these organisms have different impacts on clinical outcome and require different treatment and management approaches. It is therefore important to correctly identify *Acinetobacter* species. Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) has been introduced to identify a wide range of microorganisms in clinical laboratories, but only a few studies have examined its utility for identifying *Acinetobacter* species, particularly those of the non-*Acinetobacter baumannii* complex. We therefore evaluated MALDI-TOF MS for identification of *Acinetobacter* species by comparing it with sequence analysis of *rpoB* using 123 isolates of *Acinetobacter* species from blood. Of the isolates examined, we identified 106/123 (86.2%) to species, and 16/123 (13.0%) could only be identified as acinetobacters. The identity of one isolate could not be established. Of the 106 species identified, 89/106 (84.0%) were confirmed by *rpoB* sequence analysis, and 17/106 (16.0%) were discordant. These data indicate correct identification of 89/123 (72.4%) isolates. Surprisingly, all blood culture isolates were identified as 13 species of *Acinetobacter*, and the incidence of *Acinetobacter pittii* was unexpectedly high (42/123; 34.1%) and exceeded that of *A. baumannii* (22/123; 17.9%). Although the present identification rate using MALDI-TOF MS is not acceptable for species-level identification of *Acinetobacter*, further expansion of the database should remedy this situation.

Keywords: *Acinetobacter*, blood culture isolates, MALDI-TOF MS, non-*A. baumannii* complex, *rpo*B

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Introduction

The genus *Acinetobacter* comprises 30 named and nine genomic species [1,2]. Its most clinically important representative, *A. baumannii*, has emerged as one of the most problematic pathogens for healthcare institutions worldwide because of its resistance to several antibiotics [1,3]. Phenotypic tests only identify members of the *Acinetobacter calcoaceticus*–*A. baumannii* complex [4–7], in contrast, several molecular methods are more effective. Among these, amplified 16S rRNA gene restriction analysis, amplified fragment length polymorphism and 16S rRNA and RNA polymerase β-subunit (*rpoB*) gene sequence analyses are most frequently used [1,8]. The most effective technique may be *rpoB* gene sequence analysis because of abundant *rpoB* polymorphisms [9], and it has facilitated species identification [8,9]. Species other than

A. baumannii, such as *Acinetobacter pittii*, *Acinetobacter nosocomialis*, *Acinetobacter ursingii* and *Acinetobacter haemolyticus*, are also important nosocomial pathogens [4,7,10,11].

Carbapenems play an important role in treating *Acinetobacter* infections; however, carbapenem-resistant and multidrug-resistant *A. baumannii* strains have spread worldwide in the past two decades [3]. The expression of OXA-type class D carbapenemases, which are endogenous or acquired, is activated by promoter sequences located within IS*Aba* insertion sequences [12,13]. Metallo-β-lactamases (MBLs) also confer carbapenem resistance. The bla_{IMP-1} gene, which encodes an MBL, was frequently detected in carbapenem-resistant non-*A. baumannii* isolates [14–16]. The β-lactamases act synergistically with other mechanisms of drug resistance, including alteration of drug permeability and efflux pumps. Colistin is useful for treating *A. baumannii* infections as well, and most *Acinetobacter* species are susceptible to it. However, *Acinetobacter* genomic species 13BJ is intrinsically colistin resistant [4,17].

Although molecular genetic methods identify *Acinetobacter* species, they are typically too labour-intensive for use in clinical laboratories. Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) is increasingly used to identify a wide range of microorganisms in clinical laboratories, including Gram-positive and Gram-negative bacteria, yeasts and even filamentous fungi [18]. It is a rapid and inexpensive alternative to molecular genetic identification and offers equivalent accuracy [19]. Moreover, complete bacterial identification using MALDI-TOF MS is more cost-effective than conventional methods [20,21]. Nevertheless, some non-fermenting Gram-negative bacilli, including *Acinetobacter* species, have been misidentified because of an incomplete database [19]. Further, few published studies are available regarding the application of MALDI-TOF MS to *Acinetobacter* species.

Therefore, the purpose of the present study was to evaluate the ability of MALDI-TOF MS to identify *Acinetobacter* species. We report here a comparison of MALDI-TOF MS with *rpoB* sequence analysis of *Acinetobacter* strains isolated from blood cultures.

Materials and Methods

Bacterial isolates

This study included 123 consecutive blood culture isolates of *Acinetobacter* species collected between April 2003 and March 2011 from 123 patients in two University Hospitals in Japan. Isolates were routinely identified as *Acinetobacter* species using phenotypic methods, the Microscan Walkaway System (Siemens Healthcare Diagnostics Japan, Tokyo, Japan) and the Vitek 2 System (Sysmex-bioMérieux Japan, Kobe, Japan). The 16 type and reference strains of *Acinetobacter* used in this study were as follows: *A. baumannii* ATCC17978, *A. baylyi* KCTC12413^T, *A. bereziniae* LMG1003^T, *A. calcoaceticus* KCTC2357^T, *A. guillouiae* LMG988^T, *A. grimontii* KCTC12416^T, *A. junii* KCTC12406^T, *A. johnsonii* KCTC12405^T, *A. lwoffii* KCTC12407^T, *A. nosocomialis* LMG10619^T, *A. oleivorans* KCTC23045^T, *A. pittii* LMG1035^T, *A. radioresistens* NBRC102413^T, *A. soli* KCTC22184^T, *A. ursingii* KCTC12410^T and *Acinetobacter* gen. sp. 14BJ LMG10627. All strains were stored at −80°C, precultured for 12–24 h, and cultured aerobically overnight on 5% sheep blood agar at 37°C.

Identification of *Acinetobacter* species

Species identification of isolates was performed by partial sequence analysis of *rpoB*, using the primers Ac696F and Ac1093R [8]. All isolates were considered correctly identified when the *rpoB* sequence yielded ≥98% identity with the closest species sequence match in the GenBank database. Identification of *A. baumannii* was confirmed by PCR amplification of $bla_{OXA-51-like}$ [12]. A neighbour-joining (NJ) tree was constructed using the MEGA software, version 5 [22].

MALDI–TOF MS

Strains were extracted as described [18]. A sample of each colony was suspended in 300 μL distilled water and adjusted to McFarland standard 2, and 900 μL absolute ethanol was added. The suspension was vortexed vigorously and centrifuged at 20 000 ***g*** for 5 min. The supernatant was discarded, and the pellet was dried at 55°C for at least 30 min. Ten microlitres of 70% formic acid (Wako Pure Chemical Industries, Osaka, Japan) was then added and thoroughly mixed by pipetting. Next, 10 μL of acetonitrile (Wako) was added, and the sample was centrifuged again at 20 000 ***g*** for 5 min, and then 1 μL of supernatant was placed onto a stainless steel target plate (Bruker Daltonik GmbH, Lepizig, Germany) and dried for c.10 min at room temperature. Finally, 1.5 μL of matrix solution, comprising a saturated solution of α-cyano-4-hydrocinnamic acid (Bruker Daltonik) in 50% acetonitrile and 2.5% trifluoroacetic acid (Wako), was applied to the samples and co-crystallized at room temperature for 10 min.

The samples prepared using the standard extraction method described above were applied to a MicroFlex LT mass spectrometer (Bruker Daltonik). Each measurement was performed once for each culture. *Escherichia coli* DH5α was used as a quality control for each experiment, as recommended by the manufacturer.

Data analysis

The log score identification criteria recommended by the manufacturer (Bruker Daltonik) were used as follows: ≥2.300, reliable species; 2.00–2.299, probable species; 1.700–1.999, genus; and <1.700, unreliable. Duplicate experiments were performed. For identifying genus and species, the least stringent identification criterion was used. If the genus of one strain was identified in the first experiment and the species in the second, the genus was used for analysis. If the results of two experiments were different, such as genus or unreliable identification, the unreliable identification result was used. A dendrogram was constructed using the correlation distance measure and the average linkage algorithm settings of the BIOTYPER 3.0 software (Bruker Daltonik).

Results

Identification of *Acinetobacter* species

Thirteen species were identified according to *rpoB* sequence analysis (Table 1). The identities of the 123 isolates were as follows: *A. pittii* (n = 42); *A. baumannii* (n = 22); *A. nosocomialis* (n = 19); *A. ursingii* (n = 15); *A. grimontii* (n = 8); *A. oleivorans* (n = 4); *A. bereziniae* (n = 2); *A. soli* (n = 2); *A. johnsonii* (n = 1); *A. junii* (n = 1); *A. baylyi* (n = 1); *A. radioresistens* (n = 1); and *Acinetobacter* gen. sp. 14BJ (n = 1). The sequences of four strains were <98% identical to the *rpoB* sequence of any species in the GenBank database and could not be reliably identified; of these four strains, three strains were 96% identical and one strain was 97% identical to the *rpoB* sequence of *A. baumannii*.

TABLE 1. Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF) identification of the 123 isolates from patients' blood samples, showing scores

rpoB (zone I) sequencing	MALDI-TOF identification	Score value	Number of isolates	*bla*$_{OXA-51}$
A. pittii (n = 42)	*A. pittii*	≥2.00	37 (21)[a]	–
		1.700–1.999	5	
A. baumannii (n = 22)	*A. baumannii*	≥2.00	21 (10)	+
		1.700–1.999	1	
A. nosocomialis (n = 20)	*A. nosocomialis*	≥2.00	14 (9)	–
	A. baumannii	≥2.00	3 (2)	
		1.700–1.999	2	
A. ursingii (n = 15)	*A. ursingii*	≥2.00	14 (2)	–
		1.700–1.999	1	
A. grimontii (n = 8)	*A. junii*	≥2.00	8 (6)	–
A. oleivorans (n = 4)	*A. calcoaceticus*	1.700–1.999	3	–
	A. pittii	1.700–1.999	1	
A. bereziniae (n = 2)	*A. guillouiae*	≥2.00	1 (0)	–
		1.700–1.999	1	
A. soli (n = 2)	*A. baylyi*	≥2.00	1 (0)	–
		1.700–1.999	1	
A. johnsonii (n = 1)	*A. johnsonii*	≥2.00	1 (1)	–
A. junii (n = 1)	*A. junii*	≥2.00	1 (0)	–
A. baylyi (n = 1)	*A. guillouiae*	≥2.00	1 (1)	–
A. radioresistens (n = 1)	*A. radioresistens*	≥2.00	1 (1)	–
Acinetobacter gen. sp. 14BJ (n = 1)	Not reliable identification	<1.700	1	–
Not reliable identification (n = 4)	*A. baumannii*	≥2.00	3 (0)	–
		1.700–1.999	1	

[a]The number of isolates with score ≥2.3.

The dendrogram indicated that these four strains comprise a cluster, which is closely related to a cluster represented by *A. nosocomialis*: LMG10619 (Fig. 1). Further analysis of these four isolates is planned. PCR analysis detected the *bla*$_{OXA-51\text{-like}}$ gene in 22 of the *A. baumannii* strains; PCR analysis of the other 101 strains failed to detect a product (Table 1).

MALDI-TOF MS

MALDI-TOF MS analyses yielded scores of $\chi \geq 2.3$, 53/123 (43.1%); $2.00 \leq \chi < 2.3$, 53/123 (43.1%); $1.7 \leq \chi < 2$, 16/123 (13.0%); and $\chi < 1.7$, 1/123 (0.8%) for reliable species, probable species, genus and unreliable identifications, respectively. Based on their *rpoB* sequences, 106 isolates with scores $\chi \geq 2.0$, 89/106 (84.0%) were confirmed at the species level and 17/106 (16.0%) were not (Table 1). Hence, species identification of 89/123 (72.4%) was achieved. Interestingly, seven of the 16 unconfirmed results scoring $1.7 \leq \chi < 2$ were confirmed by their *rpoB* sequences. Therefore, the overall level of concordance between MALDI-TOF MS (valid and invalid results) and *rpoB* sequence analysis at the species level was 78.0% (96/123).

Among the 17 discordant results for species, the three isolates identified as *A. baumannii* by MALDI-TOF MS, in which *bla*$_{OXA-51\text{-like}}$ sequences were undetectable, were identified as *A. nosocomialis* according to *rpoB* sequence analysis. The eight isolates identified as *A. junii* by MALDI-TOF MS were identified instead as *A. grimontii* isolates according to their *rpoB* sequences. This discrepancy may be attributed to the synonymy of *A. grimontii* and *A. junii* [23]. Sequence analysis of *rpoB* revealed that the isolates of *A. guillouiae* and *A. baylyi* (one each) were actually *A. bereziniae* and *A. soli*, respectively. This discrepancy was caused their absence from the database.

A dendrogram was created using the 123 isolates and 16 reference strains (Fig. 2). Three *A. nosocomialis* isolates erroneously identified as *A. baumannii* with scores of $\chi \geq 2.0$ by MALDI-TOF MS cluster with *A. nosocomialis* LMG10619. This cluster includes two *A. nosocomialis* isolates erroneously identified as *A. baumannii* (MALDI-TOF MS scores, $1.7 \leq \chi < 2$). These erroneous identifications were caused by inaccurate taxonomic assignment of the given spectra within the Bruker database. One *A. baylyi* isolate erroneously identified as *A. guillouiae* clusters with reference strains *A. guillouiae* LMG988 and *A. bereziniae* LMG1003. *Acinetobacter baylyi* is known to be transformable by DNA from other *Acinetobacter* species [24]. In some cases, it has picked up the *rpoB* gene from *A. guillouiae* [24]. These intragenic recombination events may have caused this erroneous identification. Four isolates, which were not reliably identified by *rpoB* sequence analysis, represent a cluster with no reference strain. Two were identified as *A. baumannii* (MALDI-TOF MS, $\chi \geq 2.0$) and the others as *A. baumannii* ($1.7 \leq \chi < 2$).

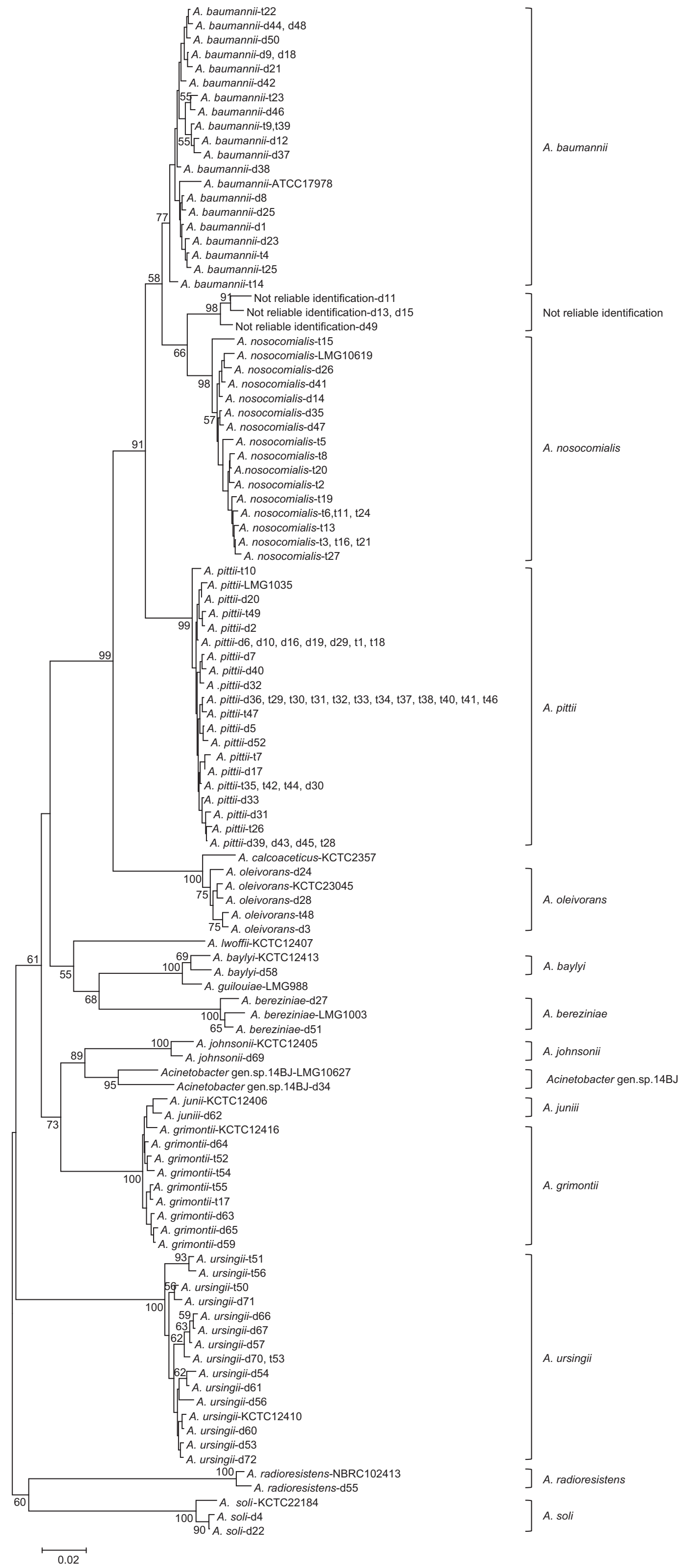

FIG. 1. Dendrogram generated from partial *rpoB* sequences for all isolates and reference strains. The scale bar indicates a genetic distance of 0.02, and the numbers shown next to each node represent bootstrap values (1000 replicates). The strain numbers next to a species name indicate that sequence types of the isolates with those strain numbers are identical to that of the species.

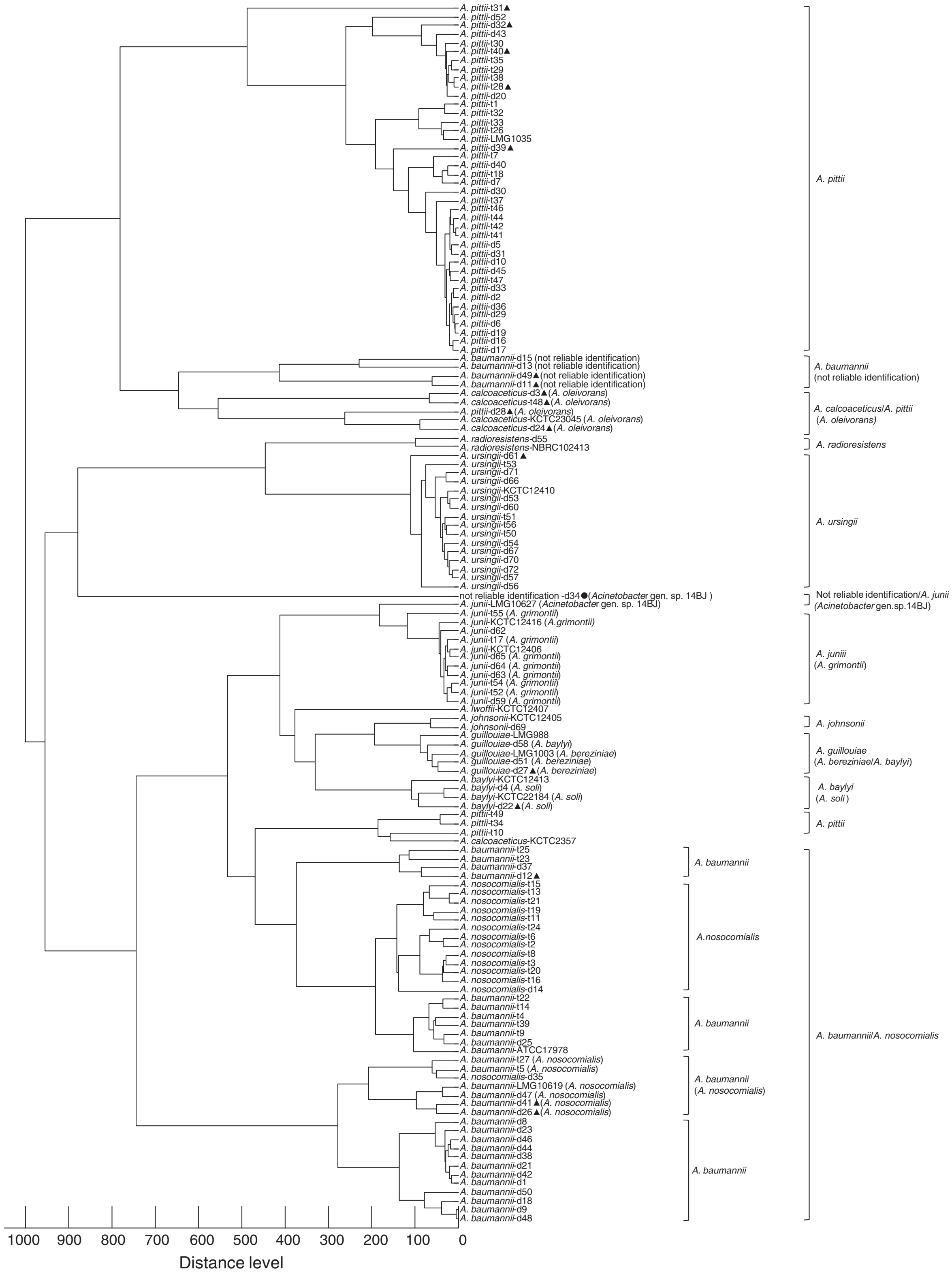

FIG. 2. Dendrogram generated using the matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) -specific protein signatures for all isolates and reference strains from the MALDI Biotyper 3.0 database. The species names in parentheses indicate identifications that are discordant with the *rpoB* sequence. Distance values are relative and normalized to a maximum value of 1000. ▲ indicates a score of 1.7 $\leq \chi <$ 2 (genus identification) and ● indicates a score of $\chi <$1.7.

Discussion

MALDI-TOF MS is a promising clinical microbiological laboratory technique that can rapidly identify a wide range of bacterial and fungal species [18,19]. MALDI-TOF MS correctly identifies the species of 85% of routine clinical isolates [19]. In the present study, however, valid species identification was achieved for only 89/123 (72.4%) of the isolates. Discordant results at the species level were obtained for 17/123 (13.8%) of the isolates and were mainly caused by recent changes in the taxonomy of a given species, the absence of the species name, or inaccurate taxonomic assignment of a given spectrum in the MALDI-TOF MS database. These taxonomic discordances were corrected by updating the database.

We selected 16 type and reference strains for the present study to create a set of reference spectra to complement the MALDI-TOF MS database, and spectra from 123 isolates were reanalysed using the local database. Hence, the original sensitivity was improved from 74.8% to 82.4% (Table 2). This update improved identification of genus (16/123, 13.0%) as well. Specifically, the discordances and errors were largely caused by an incomplete database. An extensive database is critical for accurate identification of *Acinetobacter* species by MALDI-TOF MS.

In the present study, *rpoB*-sequence analysis assigned the 123 isolates to 13 species and could not reliably identify them all (Fig. 1). In contrast, MALDI-TOF MS analysis assigned the 123 isolates to nine species and did not reliably identify them all (Fig. 2). These results indicate that the discriminatory power of MALDI-TOF MS is less than that of sequence analysis of *rpoB*; however, the latter method is laborious and not suitable for clinical laboratories. The main advantages of MALDI-TOF MS are that species can be routinely identified faster than sequencing *rpoB*, and the costs of consumables are lower. Further, MALDI-TOF MS spectra may permit an educated guess regarding the identity of an unknown.

TABLE 2. 2 × 2 contingency tables based on the original results (a) and the results acquired after updating the matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) database (b)

	rpoB gene identification	
	Reliable identification	Not reliable identification
(a) MALDI-TOF MS-based identification		
Valid and correct identification	89	0
Non-valid and discordant identification	30	4
	Sensitivity = 74.8%	Specificity = 100.0%
(b) MALDI-TOF MS-based identification		
Valid and correct identification	98	0
Non-valid and discordant identification	21	4
	Sensitivity = 82.4%	Specificity = 100.0%

The clinical relevance of *Acinetobacter* species as human pathogens, other than *A. baumannii*, has not been sufficiently assessed because of the scarcity of simple phenotypic tests used in diagnostic laboratories. In the present study, 78.8% (97/123) blood culture isolates were identified as 12 species of *Acinetobacter* other than *A. baumannii*. Six of the 12 species, *A. ursingii*, *A. grimontii*, *A. johnsonii*, *A. junii*, *A. baylyi* and *A. radioresistens*, were identified as *A. lwoffii* according to phenotypic tests, and the other six species, *A. pittii*, *A. nosocomialis*, *A. oleivorans*, *A. bereziniae*, *A. soli* and *Acinetobacter* gen. sp. 14BJ, and *A. baumannii* were identified as *A. baumannii* by phenotypic identification. Surprisingly, the incidence of *A. pittii* was high, 42/123 (34.1%), and exceeded that of *A. baumannii*, 22/123 (17.9%). *Acinetobacter pittii* is implicated in endocarditis and can cause life-threatening infections, as do *A. baumannii*, *A. nosocomialis*, *A. johnsonii*, *A. lwoffii* and *A. beijerinckii* [4,25,26]. The relatively high incidence of *A. ursingii*, which accounted for 15/123 (12.2%) of isolates, was unexpected but agrees with observations from hospitals in the UK, the Netherlands and Northern Ireland [4,27,28]. This organism infects the bloodstream of hospitalized patients [7,11,29], and it has been associated with a nosocomial outbreak of fatal bloodstream infections in a neonatal intensive care unit [10]. Infections caused by these organisms have different impacts on clinical outcome and require different treatment and management approaches [30]. It is therefore extremely important to correctly identify *Acinetobacter* species, including non-*A. baumannii* isolates.

In conclusion, MALDI-TOF MS is currently not adequate for species-level identification of *Acinetobacter*. However, our present studies show that further expansion of the database to include *Acinetobacter* species other than *A. baumannii* will make MALDI-TOF MS an efficient method for identification of nosocomial *Acinetobacter* species. Some *Acinetobacter* species other than *A. baumannii*, particularly *A. pittii*, *A. nosocomialis* and *A. ursingii*, have been associated with outbreaks [10,28], suggesting that they may become an increasingly important healthcare problem. We expect that MALDI-TOF MS will provide a useful method for clinical laboratories to identify *Acinetobacter*.

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Transparency Declaration

The authors have no conflicts of interest.

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